MOLECULAR AND CELLULAR BIOLOGY, Oct. 1987, p. 3538–3547
0270-7306/87/103538-10$02.00/0

Vol. 7, No. 10

Long-Term Effects of Estrogen on Avian Liver: Estrogen-Inducible Switch in Expression of Nuclear, Hormone-Binding Proteins

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Received 24 March 1987/Accepted 20 July 1987

The stimulation of chicks or embryos with estrogen results in transient, hepatic expression of the vitellogenin gene, as well as long-term, propagatable alterations in the rapidity with which the gene can be reactivated. We examined the possibility that nuclear, type II estrogen-binding sites are involved in this long-term change in response characteristics. We demonstrate that the primary induction kinetics of type II sites in embryos and chicks correlated with the expression of the vitellogenin gene and that once their induction was triggered by estrogen, they accumulated, were propagated, and persisted for months after withdrawal of the hormone. We also show that their accumulation in the embryo was accompanied by prolonged expression of both the vitellogenin and very low-density apolipoprotein II genes, in the absence of elevated levels of type I receptor, and that the type II sites, like the classical receptor, appear to be preferentially associated with active or potentially active chromatin. Finally, we describe a regulatory mechanism, tested by computer modelling, that simulated the behavioral characteristics of these nuclear estrogen-binding sites and which may explain their role in mediating the long-term effects of estrogen.

Estrogen exerts its effects on gene expression primarily through interaction with the classical, nuclear high-affinity ($K_d = 10^{-9} - 10^{-10}$ M), low-capacity type I receptor (12, 13, 16). The receptor-hormone complex has been shown to bind to DNA at specific sites or response elements in the proximity of hormonally regulated genes and to enhance their expression in a variety of gene transfer experiments (29). However, a second, lower-affinity, nuclear, estrogen-binding protein, designated as the type II binding site (22), has also been identified in a number of target tissues and has been shown to be elevated in transformed cells derived from these tissues (6, 10, 22, 36, 37, 40, 41). It has been suggested that these sites may play a currently unspecified role in the mitotic response of estrogen target tissues (22, 23). The type II binding sites differ from the classical estrogen receptor in several respects. (i) They display a different range of affinities for a variety of steroids. (ii) They are extremely sensitive to reducing agents, while the classical type I receptor is stabilized by them. (iii) They are much more resistant to salt extraction from the nucleus than is the classical receptor (24, 33).

We have demonstrated recently that a lower-affinity, estrogen-binding component with the characteristics of the type II site can be induced in the human hepatoma cell line HepG2 with low concentrations of estrogen. Once induced, in contrast to the type I receptor, these sites are propagated in daughter cells for 15 or 16 cell generations in the absence of hormone (37, 38). This behavior prompted us to suggest that the type II sites could play a role in mediating the long-term effects that estrogen is known to have on some of its target tissues. We have now examined this possibility by using avian liver as a model.

Activation of vitellogenesis in avian liver is one of the most thoroughly characterized in vivo models in which specific, long-term effects of estrogen on gene expression have been demonstrated. The onset of vitellogenesis in the maturing female chick involves hepatic synthesis of a group of yolk proteins (1, 31). The genes encoding these proteins are expressed at various levels and exhibit various degrees of dependence on estrogen. Two of the major yolk proteins, apoVLDLII and vitellogenin, are specified by genes whose expression is completely estrogen dependent (9, 15, 43).

Under normal circumstances, the apoVLDLII and vitellogenin genes are expressed only in laying hens. However, the transcription of both can be activated in birds of either sex by pharmacological doses of estrogen. This can be accomplished at any stage after day 8 of embryogenesis in the case of the apoVLDLII gene, but to elicit detectable expression of the vitellogenin gene, the embryos must be at least 11 days old (11, 20). In chicks or adult roosters, the genes respond rapidly to estrogen (mature mRNA being detectable within 30 min to 1 h of stimulation), but the initial rate of expression of both is relatively low. In response to primary stimulation with hormone, the maximal rate of accumulation of apoVLDLII mRNA is not attained until 3 to 4 h after stimulation, while that of vitellogenin mRNA is delayed still further, lagging behind apoVLDLII mRNA accumulation by 5 to 6 h (9, 15, 43). The concentrations of both apoVLDLII and vitellogenin mRNAs peak at approximately 3 days and decline to essentially preinduction levels within 2 weeks of the administration of hormone. In addition to this short-term response, primary stimulation with estrogen also has a long-lasting effect on the kinetics with which maximal expression of the genes can be restored. Restimulation of pretreated birds up to several months after the initial response has ceased results in a more rapid activation of both genes that is particularly apparent in the case of the vitellogenin gene (7, 9, 15). This memory effect is observed even if the initial stimulation with estrogen is carried out in ovo, clearly demonstrating that it can be propagated during growth of the liver in the absence of hormone (3).

In the studies described here, we have examined the kinetics of the induction and disappearance of both type I and type II nuclear estrogen-binding sites following primary stimulation of adult birds, as well as embryos at various stages of development. The results demonstrate that (i) the

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induction of type II sites in chicks and adult birds exhibits a lag of several hours, but once induced the sites remain at significantly elevated levels for at least several months; (ii) during embryogenesis, the ability to induce type II sites lags behind induction of type I sites by approximately 2 days; (iii) a single dose of estrogen administered at day 13 of embryogenesis causes only transient induction of type I sites that lasts 3 to 4 days but results in a continuous increase in the number of type II sites per cell until hatching; and (iv) the accumulation of type II sites after induction at day 13 is accompanied by the expression of the apoVLDLII and vitellogenin genes that also continues until hatching, despite the fact that nuclear type I receptor levels have declined to control levels 4 days earlier. Finally, we present a working model, assessed by computer simulation, that attempts to explain the behavior of type II binding sites and the regulatory role that they may play.

MATERIALS AND METHODS

Animals and treatment. White leghorn embryos were treated in ovo by the injection of estrogen (1.25 mg of 17β-estradiol in 50 μl of propylene glycol) into the yolk sac. Male chicks between 3 weeks and 2 months of age received 17β-estradiol (25 mg/kg of body weight at a concentration of 25 mg/ml in propylene glycol) by injection into the thigh muscle.

Receptor assays. Specific binding of 17β-estradiol to nuclear type I and type II binding sites was carried out on charcoal-stripped, 0.4 M KCl extracts as described previously (19), with the following modifications (38). Reducing agents were omitted from all stages of nucleus isolation and salt extraction. The liberation of type II sites was accomplished by subjecting all nucleus preparations to at least two freeze-thaw cycles in extraction buffer (400 mM KCl, 10 mM EDTA, 10 mM Tris hydrochloride, pH 7.4). Levels of type II receptors were determined by measuring the difference in estrogen-specific binding at 37°C with or without 0.1 mM dithiothreitol.

DNase I treatment of nuclei. Nuclei prepared as previously described (19) were suspended in digestion buffer (3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM NaCl, 10 mM Tris hydrochloride, pH 7.2) at a concentration equivalent to 1 mg/ml in DNA. Samples were divided into three aliquots, one of which was used as a control, while the others were incubated at 37°C for 10 min to allow digestion by either endogenous nucleases alone or with the addition of DNase I (50 U/mg of DNA). The reaction was stopped with EDTA (final concentration 10 mM), the nuclei were centrifuged at 5,000 × *g* for 10 min, and the supernatant was removed. After being washed in digestion buffer plus EDTA (10 mM), the nuclei were suspended at a concentration of 1.5 mg of DNA/ml in extraction buffer and kept on ice for at least 30 min with frequent mixing. Extractable estrogen receptors were recovered in the supernatant after centrifugation at 10,000 × *g* for 10 min at 4°C.

Analysis of RNA. Total RNA was prepared by a guanidine hydrochloride extraction procedure (8). Samples of each RNA preparation (20 μg) were glyoxalated and electrophoresed through 1.5% agarose gels in phosphate buffer (10 mM, pH 7) (26). The RNA was transferred onto Biodyne nylon membrane (Pall) and hybridized with 2×10^7 cpm of nick-translated apoVLDLII or vitellogenin cDNA clones (specific activity, 4×10^7 cpm/μg) under standard conditions (39).

Run-on transcription assays. Nuclei were prepared (30) 5 or 7 days following the administration of estrogen to day 13 embryos and either used immediately or stored at −70°C. Run-on transcription assays were performed as described for 40 min at 26°C (30). Each incubation of 200 μl contained 1×10^7 to 2×10^7 nuclei, 1 U of RNasin per μl, and 100 μCi of [α-^{32}P]UTP (specific activity, 752 Ci/mmol). Reactions were terminated by the addition of DNase I (RNase free; Bethesda Research Laboratories), and labeled RNA was isolated (25).

Specific gene transcripts were quantified by hybridization to filter-bound DNA. The DNA clones used were pSVT13 and pSAP34, which are derived from vitellogenin and apoVLDLII mRNAs, respectively. Both were the generous gift of J. Burch. Throughout the hybridization selection experiments, pGEM1 DNA (Promega Biotec) was used as a nonspecific control. Preparation of the DNA-diazophenylthioether cellulose filters was as outlined previously (35). Hybridization, washing, and elution of bound, labeled RNA was as previously described (25). Nonspecific hybridization to each filter was determined by carrying out transcription assays with liver nuclei from untreated embryos at various stages of development and with liver and kidney nuclei from mature roosters. The relative rates of transcription in estrogen-treated embryos were calculated in terms of parts per million, after subtraction of control values. Prior to correction for the size of the probe used and the size of its corresponding gene, these values ranged from 38 ppm to 120 ppm. Total incorporation into RNA per assay ranged from 1×10^7 to 5×10^7 dpm, all of which was used for each hybridization selection. Throughout the analyses, tritiated, sense-strand RNAs produced from pSVT13 and pSAP34 by transcription with SP6 polymerase were used to normalize for differences in hybridization efficiency. The efficiency ranged from 30 to 35%.

Simulation of the regulation of type II binding sites. The model on which the simulation is based is represented in Fig. 4A.

The rate of accumulation of type II sites is described by equation 1,

$$\frac{dT2}{dt} = k_1 A - k_2 T2 \quad (1)$$

where k_1 and k_2 are rate constants for posttranscriptional production and decay of type II sites (T2), respectively, and A represents the activity of the type II gene. The activity of the gene is presumed to be determined by the extent to which two response elements, RE1 and RE2, are occupied by type I receptor-estrogen complex (T1) and type II receptor (T2), respectively. Thus,

$$A = a_1 \cdot \frac{T1}{K1 + T1} + a_2 \cdot \frac{T2}{K2 + T2} \quad (2)$$

where a_1 and a_2 are coefficients of activation attributable to the respective response elements RE1 and RE2. Equation 2 implies saturable binding interactions between T1 and RE1 and between T2 and RE2, each characterized by dissociation constants $K1$ and $K2$. The combination of equations 1 and 2 yields equation 3, which expresses the rate of change of T2 with time.

$$\frac{dT2}{dt} = k_1 a_1 \cdot \frac{T1}{K1 + T1} + k_1 a_2 \cdot \frac{T2}{K2 + T2} - k_2 T2 \quad (3)$$

The first term represents stimulation of the gene by type I

receptor-hormone complexes (T1); the second term represents closed-loop, positive-feedback activation of the gene by type II receptors (T2); and the last term represents first-order decay of these sites. The solution to equation 3 was accomplished by inversion, followed by integration to give time (t) as a function of type II concentration (T2). Upon inversion, equation 3 can be written as indicated in equation 4, where the term S (stimulation by T1) has been used to replace $k_1a_1 \cdot T1/(K1 + T1)$.

$$\frac{dt}{d\mathrm{T2}} = \frac{1}{\mathrm{S} + \dfrac{k_1a_2\mathrm{T2}}{K2 + \mathrm{T2}} - k_2\mathrm{T2}} \tag{4}$$

Multiplication of the numerator and denominator of equation 4 by K2 + T2, plus use of the substitutions $U = K2 + T2$, $dT2 = dU$, $\alpha_1 = (S + k_1a_1 + k_2K2)/k_2$ and $\alpha_0 = k_1a_2K2/k_2$, yields equation 5.

$$dt = -\frac{1}{k_2}\left(\frac{U\,dU}{U^2 - \alpha_1 U + \alpha_0}\right) \tag{5}$$

Equation 5 is readily integrated by the method of partial fractions to yield equation 6, describing the relationship between increments in t (Δt) and increments in T2 (ΔT2).

Expressions for the values of X and Y in equation 6 are given by equations 7 and 8.

$$\Delta t = \frac{1}{k_2} \cdot \frac{1}{(Y - X)}\left[X \ln\left(1 + \frac{\Delta\mathrm{T2}}{\mathrm{T2} + K2 + X}\right) - Y \ln\left(1 + \frac{\Delta\mathrm{T2}}{\mathrm{T2} + K2 + Y}\right)\right] \tag{6}$$

$$X = \frac{-\alpha_1 - \sqrt{\alpha_1^2 - 4\alpha_0}}{2} \tag{7}$$

$$Y = \frac{\alpha_1 + \sqrt{\alpha_1^2 - 4\alpha_0}}{2} - \alpha_1 \tag{8}$$

Thus, the parameters X and Y are constants over a particular interval in ΔT2, and their values are determined by S and the other dissociation constants and rate constants of the model. The above equation was used to simulate the time course of T2 accumulation by incrementing T2 and calculating the corresponding times t. The simulation was performed in BASIC by using a Perkin-Elmer model 7500 minicomputer. Graphic representation of the results was obtained by using software provided by the manufacturer, details of which will be provided on request. The concentration of type I receptor-estrogen complex, represented by the term T1 in equation 1, was varied by using the formula $T1 = 0.01 + t/(7 + t)$, where t is the accumulated time. Thus, the time course and extent of type I receptor accumulation was approximated by a rectangular hyperbola to simulate experimental observations. Within the program, the option existed to set $S = 0$ at predetermined times in order to simulate the removal of estrogen.

RESULTS

Accumulation and persistence of type II estrogen receptors in rooster liver. After primary administration of estrogen to male chicks or roosters, the kinetics of accumulation of

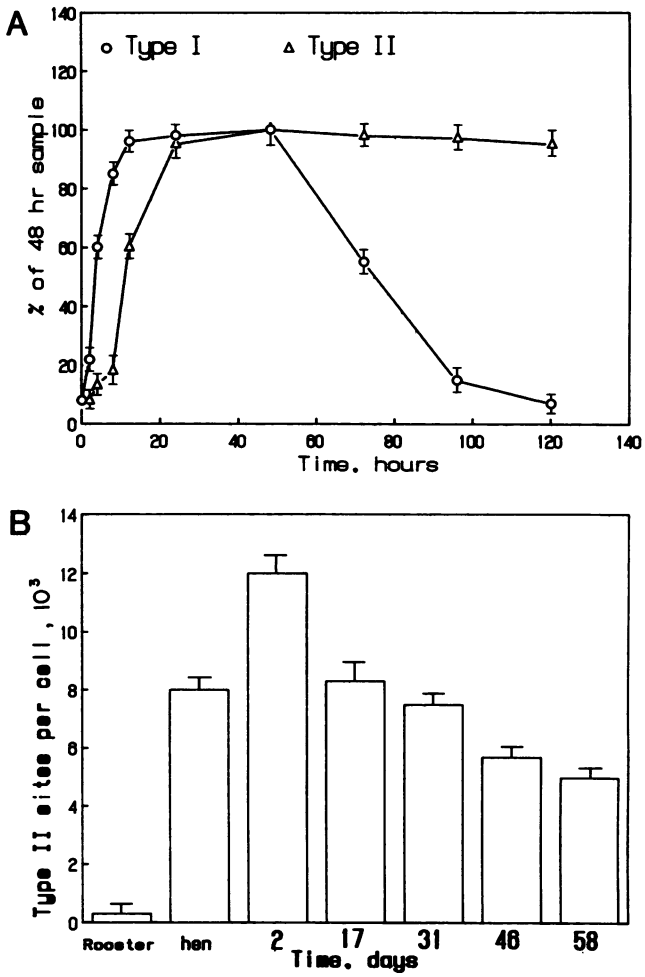

FIG. 1. (A) Kinetics of induction of nuclear estrogen-specific binding sites in the livers of male chicks after a single intramuscular injection of 17β-estradiol. Specific binding of [^{3}H]17β-estradiol to high- and low-affinity binding sites was estimated in charcoal-stripped 0.4 M KCl nuclear extracts. Induction of type I (high-affinity) and type II (low-affinity) sites is plotted as a function of time after the administration of hormone. The levels of binding sites have been expressed as a percentage of the fully induced levels reached 48 h after injection (3,000 and 12,000 sites per cell for high- and low-affinity sites, respectively). (B) Persistence of type II nuclear estrogen-specific binding sites in the livers of male chicks after primary stimulation. The numbers of type II nuclear binding sites per cell are displayed for untreated roosters, laying hens, and for estrogen-treated male chicks at specified times (in days) after the administration of a single intramuscular injection of hormone. Each bar represents the average value (± the standard error of the mean [SEM]) of type II binding sites for at least three different animals. Specific binding of [^{3}H]17β-estradiol to nuclear type II binding sites was carried out by using charcoal-stripped salt extracts.

nuclear type I receptors precede increases in the rate of apoVLDLII and vitellogenin mRNA accumulation by 1 to 2 and 6 to 8 h, respectively (14, 34, 43). This observation raises the possibility that factors in addition to type I receptor play a role in determining the maximal rate of expression of at least the vitellogenin gene. In experiments summarized in Fig. 1A, we determined the kinetics of induction of both type I and type II nuclear binding sites after primary stimulation of male chicks with estrogen under conditions that are

TABLE 1. Release of nuclear estrogen-specific binding sites from nuclei by incubation with nuclease[a]

Nuclear fraction	No. of sites/cell in sample:					
	I		II		III	
	Type I	Type II	Type I	Type II	Type I	Type II
Supernatant 1	ND	ND	512 ± 45	ND	2,100 ± 126	ND
Supernatant 2	ND	ND	319 ± 40	ND	560 ± 42	150 ± 26
Salt extract	2,460 ± 150	524 ± 110	1,880 ± 170	2,860 ± 170	311 ± 25	10,610 ± 210
Salt + freeze-thaw	439 ± 40	11,920 ± 280	198 ± 15	9,950 ± 210	212 ± 10	2,130 ± 150

[a] As described in Materials and Methods, liver nuclei from male chicks treated with estrogen for 48 h were suspended in digestion buffer at a DNA concentration of 1 mg/ml and divided into three aliquots: control (I), samples maintained on ice in the presence of 10 mM EDTA; endogenous nuclease digestion (II), samples incubated at 37°C for 10 min with no additions; and DNase I digestion (III), samples incubated at 37°C for 10 min with 50 U of DNase I per ml. Digestion was stopped, the nuclei were pelleted, and the supernatants were collected (Supernatant 1). The nuclei were rinsed once in digestion buffer plus EDTA (Supernatant 2) and then extracted for 30 min at 4°C in 0.4 M KCl. The extracts were centrifuged, and the supernatants (Salt extract) were collected. The pellets were suspended in 0.4 M KCl and subjected to two cycles of freezing and thawing at −70 and 20°C. The samples were then centrifuged, and the supernatant (Salt + freeze-thaw) were collected. All fractions were assayed for type I and type II nuclear estrogen-specific binding sites. Each value is the average (± SEM) of at least three separate experiments. ND, Not detected.

identical with those used previously to examine the induction of apoVLDLII and vitellogenin mRNAs (43).

Accumulation of type II sites was delayed relative to that of type I receptor and exhibited a lag of 6 to 8 h (Fig. 1A). However, after this initial lag, the rate of accumulation of type II sites increased rapidly, so that only 4 h later they had reached 60% of fully stimulated levels. Maximal type II levels were attained between 24 and 48 h after injection of the hormone. Thus, the slow initial accumulation of type II sites is very similar to the lag seen during primary induction of vitellogenin mRNA synthesis (9, 15). It is also apparent from data in Fig. 1A that although levels of nuclear type I receptors declined to control values within 5 days after stimulation, type II sites were still at maximal levels at this time.

Examination of the levels of type II sites over a much more extended time period is shown in Fig. 1B. Within 48 h after the administration of a bolus of estrogen, type II sites peaked at approximately 12,000 sites per cell, which is 1.5 times the number observed in laying hens. They remained at maximal levels for at least 3 days, and then over the next 2 weeks, they declined to approximately the steady-state level observed in the hen. Thereafter, the number of sites per cell decreased much more slowly, so that by 8 to 9 weeks after initial stimulation, they were still present at a level approximately 15-fold higher than in an untreated rooster and approximately 65% of the level in the hen.

Mild treatment with DNase I rendered the type II sites salt extractable. The type II estrogen receptor has been shown to resist extraction by concentrations of salt as high as 2.0 M and to be associated with nuclear matrix prepared by using salt concentrations in this range (33). Its recovery in this fraction is consistent with the possibility that it is either a component of the matrix itself or is preferentially associated with active or potentially active chromatin. However, because of the extremely high salt concentrations used, the possibility remains that the recovery of type II sites in this fraction may be artifactual, as has been suggested to explain the observed association of transcriptional complexes with matrix prepared by high-salt extraction methods (27). We have found that type II sites can be extracted by moderate concentrations of salt (0.4 M), providing that the nuclei are subjected to at least two cycles of freezing and thawing during extraction (38). In experiments summarized in Table 1, we examined whether this can also be achieved by digestion with either endogenous nucleases or concentrations of DNase I that have been shown to preferentially digest active chromatin (42) and to liberate type I receptor.

Nuclei were incubated at 37°C to allow limited cleavage of DNA either by endogenous nucleases or by DNase I. As demonstrated previously (32), this treatment is adequate to liberate type I estrogen receptors into the supernatant without salt extraction. Under the conditions used, endogenous nuclease activity released approximately 30% of the type I receptors, while supplementation with DNase I resulted in the release of approximately 85%, detectable by exchange assay. Neither endogenous nucleases nor DNase I was effective in liberating type II sites from the nucleus in low salt, but to a greater or lesser degree, both rendered the type II sites extractable by 0.4 M KCl. Furthermore, the proportion that was salt extractable following digestion closely paralleled the percentage of type I receptors liberated by the treatment (Table 1), consistent with the suggestion that both are associated with the active or potentially active chromatin fraction.

Ontogeny of induction type II receptors. The developmental stage at which avian liver becomes estrogen responsive has been examined previously by monitoring the induction of the type I receptor after the administration of the hormone and activation of the apoVLDLII and vitellogenin genes (11, 20). The results indicate that acquisition of the competence to express type I receptor and to activate the apoVLDLII gene occurs at approximately the same developmental stage, i.e., between days 7 and 9. In contrast, activation of the vitellogenin gene cannot be detected until at least 2 days later. In view of the temporal correlation between the induction of type II sites and increases in the rate of accumulation of vitellogenin mRNA in the adult, we investigated whether the relatively delayed ability to activate the vitellogenin gene in the embryo was also correlated with a comparable delay in the ability to express the type II estrogen-binding site.

Embryos were injected at various stages of development, and type II sites were assayed 48 h later. This period of stimulation induces peak levels in roosters and chicks. The data presented in Fig. 2A indicate that prior to day 13 of embryogenesis, there was no significant increase upon estrogen treatment in the number of type II sites above the very low background of sites observed in control embryos (<100 sites per cell). Slight but consistent increases in type I receptor could already be detected when embryos were injected on day 9 and the livers were examined on day 11. It

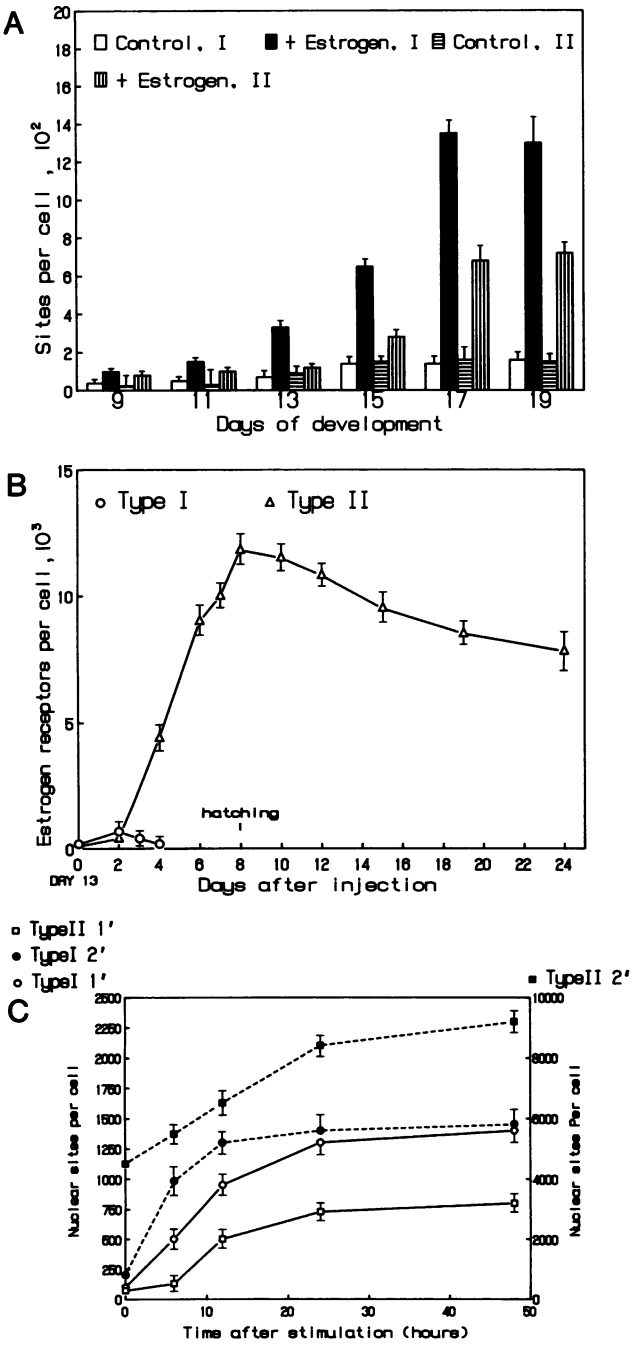

FIG. 2. (A) Levels of nuclear estrogen-specific binding sites in the livers of embryos 48 h after the administration of 17β-estradiol (1.25 mg per egg). Specific binding of [^{3}H]17β-estradiol to type I (high-affinity) and type II (low-affinity) binding sites was estimated in charcoal-stripped nuclear salt extracts. The ages of the embryos at the time of receptor assay (i.e., 48 h after stimulation) are indicated. At each time, values for nuclear type I and type II binding sites are shown for both control and estrogen-treated embryos. Each bar represents the average value (± SEM) for at least three separate experiments, each utilizing 3 to 36 embryos. (B) Levels of nuclear estrogen-specific binding sites in avian liver as a function of time after treatment with 17β-estradiol (1.25 mg per egg) on day 13 of embryogenesis. The number of type I and type II binding sites per cell, detected in charcoal-stripped nuclear salt extracts, is plotted as a function of time in days after the administration of 17β-estradiol on day 13 of embryogenesis. The date of hatching, i.e., 8 days after treatment, is indicated. Each datum point represents the mean (± SEM) of at least three separate experiments, each utilizing two to six embryos or two chicks. (C) Kinetics of primary and secondary accumulation of nuclear estrogen-specific binding sites in avian liver at day 17 of embryogenesis. Specific binding of 17β-estradiol to type I and type II binding sites was estimated in charcoal-treated nuclear salt extracts. Induction of nuclear estrogen-binding sites is plotted as a function of time in hours after the administration of hormone (1.25 mg per egg) on day 17 of embryogenesis. For secondary stimulations, the first injection of hormone (1.25 mg per egg) was performed on day 13 of embryogenesis. The levels of type II sites after secondary stimulation are indicated on the right-hand axis. Each point represents the mean (± SEM) of at least three different experiments, each utilizing at least three livers.

was not until day 13 that injection resulted in significant increases in the levels of type II sites 48 h later (250 to 300 sites per cell in treated embryos compared with 140 to 170 sites per cell in controls). Induction of the type II sites increased markedly after day 13, so that when the embryos were injected on days 15 or 17, levels 2 days later were between 700 and 800 sites per cell. Even these levels are approximately only 5% of those found at a comparable time after stimulation of adult birds or chicks. In contrast, the levels of type I receptor in day 17 to 19 embryos reached 40 to 50% of the fully induced adult levels. In subsequent experiments, we examined the extent to which this apparent developmental delay in the ability to induce the type II receptor might be attributable to much slower initial kinetics of accumulation in the embryo than in the adult.

Amplification of type II sites after decline of type I receptor. In the experiments summarized in Fig. 2B, embryos were injected at day 13 and the accumulation of both types of nuclear estrogen-binding sites was followed through to hatching. Nuclear type I receptors accumulated more slowly than in the adult and reached maximal levels of 650 sites per cell at day 15. This represents approximately 20% of the fully induced adult levels that are reached by 12 h after primary stimulation. They then declined rapidly, reaching control values by day 17. Since the data presented in the figure were derived from analyses of nuclear salt extracts, they were repeated with intact nuclei to check on the possibility that a proportion of the type I receptors might have been refractory to extraction. The results did not differ significantly from those obtained with the salt extracts (data not shown).

The decline of type I receptors to preinduction levels within 4 days of the administration of hormone strongly suggests that by this time the administered estrogen has been metabolized, or at least sequestered, so that it is unavailable for interaction with the receptor. The formal possibility also exists that significant concentrations of estrogen are still present but the ability to maintain the levels of nuclear type I receptor is overridden by some currently undefined mechanism. The latter possibility was tested by readministering estrogen on day 17 (Fig. 2C). Restimulation resulted in the rapid reinduction of nuclear type I receptor, confirming that it was still inducible by estradiol and supporting the suggestion that the concentration of available hormone from the initial dose had indeed fallen below the threshold necessary to maintain nuclear levels of this receptor.

The accumulation of type II sites after stimulation on day 13 was markedly delayed relative to the response in chicks and adult birds (Fig. 2B). The number of sites per cell increased extremely slowly for the first 48 h after the

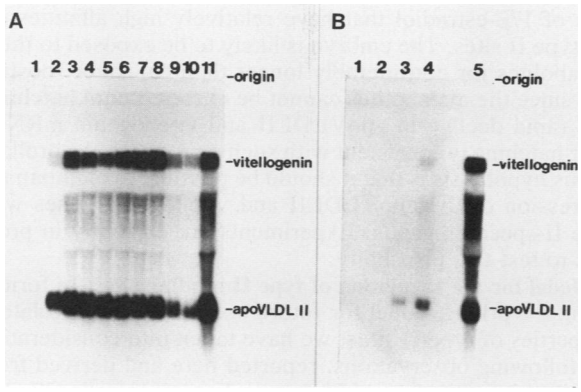

FIG. 3. (A) Levels of apoVLDLII and vitellogenin mRNA in liver after stimulation with 17β-estradiol on day 13 of embryogenesis. Total liver RNA was prepared by guanidine hydrochloride extraction from embryos, chicks, and stimulated mature roosters. Aliquots of the RNA (20 μg) were glyoxalated and electrophoresed through a 1.5% agarose gel in 10 mM phosphate buffer, pH 7.0. The RNA was transferred to a nylon membrane and hybridized with ^{32}P-labeled nick-translated apoVLDLII and vitellogenin cDNA clones (2×10^7 cpm; specific activity, 4×10^7 cpm/μg) under standard conditions, and the blot was subjected to autoradiography. Lanes: 1, day 13 control; 2 to 8, days 14 to 20 embryonic liver stimulated on days 14 to 20, taken at 1-day intervals; 9 and 10, days 2 and 4 after hatching; 11, mature rooster liver stimulated at 48 h. (B) Induction of apoVLDLII and vitellogenin mRNA in 40-day-old chicks developed from eggs primed with estrogen at day 13 of embryogenesis. Embryos were pretreated with estrogen at day 13 of embryogenesis and allowed to hatch. Liver total RNA was prepared 40 days after hatching from both pretreated and control chicks 6 h after both groups received intramuscular injections of 17β-estradiol. Aliquots of the RNA (20 μg) were glyoxalated and electrophoresed through a 1.5% agarose gel in 10 mM phosphate buffer, pH 7.0. The RNA was transferred to a nylon membrane and hybridized with nick-translated apoVLDLII and vitellogenin cDNA clones, as described for panel A. Lanes: 1, untreated control; 2, pretreated control; 3, 6 h after primary injection; 4, 6 h after secondary injection; 5, mature rooster liver stimulated at 48 h.

administration of hormone. However, between day 15 and 16 of embryogenesis, the kinetics of their accumulation accelerated dramatically, so that by day 17, there were 4,000 type II sites per cell. The number of type II sites continued to increase and reached a maximal level of approximately 12,000 sites per cell on the day of hatching (day 21). After hatching the number of sites per cell declined slowly, so that 17 days later, they had fallen to levels comparable with those in the laying hen.

Two points about the induction of nuclear type II receptors should be stressed. First, the vast majority of the sites that accumulated in response to injection at day 13 were produced during a period when nuclear type I estrogen receptor levels were declining or had already returned to control values. Second, between days 15 and 21 of embryogenesis, the DNA content of the liver and the number of sites per cell increased by 3- to 4-fold and 30-fold, respectively. Thus, the total number of type II sites in the liver was amplified by at least 100-fold during this period. On the basis of increases in DNA content, at least two cell divisions took place in the first 17 days after hatching. Consequently, by this time there was an additional amplification of two- to fourfold.

Continued expression of vitellogenin and apoVLDLII genes after decline of the type I receptors. In adult birds, the decline in expression of the apoVLDLII and vitellogenin genes after acute stimulation with a bolus of estrogen roughly parallels the fall in levels of nuclear type I receptor that occurs after metabolism of the hormone (18, 43). In experiments summarized in Fig. 3, we determined whether this is also true when embryos are stimulated at day 13.

Analysis of the levels of apoVLDLII and vitellogenin mRNAs by Northern blotting (RNA blotting) revealed that 24 h after stimulation, apoVLDLII mRNA was readily detectable but vitellogenin mRNA was barely discernible above the background level. Maximal levels of both mRNAs were reached 2 to 3 days after stimulation and remained at approximately these levels through to hatching despite the decline in the type I receptor. After hatching, the levels of both mRNAs dropped sharply, so that within 4 days, the concentration of both had decreased approximately fivefold. As reported previously (3), chicks developed from embryos pretreated in this fashion display a memory effect comparable with that following secondary stimulation of an adult bird (Fig. 3B).

It is unlikely that persistence of the mRNAs through to hatching can be attributed solely to their stability, since as pointed out above, the DNA content of the liver and yield of hepatic total RNA increased three- to fourfold between days 15 and 21 of embryogenesis and a corresponding decrease in their relative concentrations was not observed. The possibility that transcription of the genes continues in the stimulated embryos after type I receptor levels return to control values was examined more directly by nuclear run-on experiments, as described in Materials and Methods and summarized in Table 2. The results indicate that the relative rates of transcription of the vitellogenin gene in 18- and 20-day embryos that were stimulated at day 13 correspond to approximately 30 and 45%, respectively, of the rate observed in estrogen-treated roosters at the peak of a primary response. The relative rate of transcription of the apoVLDLII gene in embryos at both times was comparable with the adult rate. Thus, unlike the situation in the adult, transcription of both genes clearly continued at high levels in the embryo for several days after type I receptors returned to their preinduction values.

DISCUSSION

Induction of type II sites in male chicks. It has been demonstrated previously that primary stimulation with es-

TABLE 2. Transcriptional activity of vitellogenin and apoVLDLII genes in stimulated embryos and adults

Age	Rate (ppm) of gene transcription[a]	
	apoVLDLII	Vitellogenin
18 days	324 ± 78	836 ± 108
20 days	324 ± 79	1,166 ± 102
Adult	300 ± 28	2,640 ± 151

[a] Specific transcription was assessed as described in Materials and Methods. In summary, the transcriptional activity of each gene was calculated as follows: specific transcription (parts per million [ppm]) = [stimulated (ppm) − background (ppm)] × gene size/size of cloned fragment. Sizes of the apoVLDLII and vitellogenin genes were 2.9 and 25 kilobases, respectively, and those of the cloned apoVLDLII and vitellogenin cDNA fragments were 0.55 and 1.0 kilobases, respectively. Background was determined by hybridization with labeled RNA generated by using nuclei isolated from tissues (rooster liver and kidney) known not to express these genes. Data presented are the averages of at least four independent measurements (± SEM).

trogen alters the profile of DNase I-hypersensitive sites in regions flanking both the apoVLDLII and the vitellogenin genes and also results in demethylation at a type I receptor-binding site upstream from the vitellogenin gene (2, 4, 17, 28). In some cases, these alterations in chromatin structure persist after a primary response has ceased and can also be propagated to a limited extent after cell division in the absence of estrogen. However, the hypersensitive sites involved are lost within 7 to 8 weeks of hormone withdrawal and thus are not maintained for sufficiently long periods to explain the memory effect. Remethylation of the receptor-binding site also appears not to influence the secondary response characteristics of the vitellogenin gene (3). Our previous observation that estrogen-induced, nuclear, type II binding sites in human hepatoma cells can be propagated for 15 to 16 cell divisions in the absence of hormone (38), raises the possibility that a corresponding site plays a role in mediating the long-term effects of the hormone on avian liver.

Examination of the primary induction kinetics of hepatic type II sites in roosters revealed that they exhibit a lag similar to that displayed by the vitellogenin gene upon primary activation and that their accumulation is delayed by several hours relative to the classical, high-affinity, type I receptor. In addition, unlike the type I receptor, which accumulated and declined over a period of approximately 5 to 6 days, the type II binding sites were still present at 65 to 70% of the levels in laying hens 8 to 9 weeks after stimulation. This rate of decrease approximately parallels the decline in the magnitude of the memory response (3).

Induction of type II sites during embryogenesis. The expression of the apoVLDLII gene can be detected in estrogen-treated embryos 2 to 3 days prior to the expression of the vitellogenin gene. We found in the present studies that the induction of type II sites also could not be detected until 2 to 3 days after the first consistently detectable increase in type I levels. It is apparent from the results of experiments presented in Fig. 2B that these observations are the consequence not necessarily of a delay in the ability to express type II sites but of very low initial rates of accumulation of these sites in the embryo that result in a prolonged lag phase. We have observed previously during studies with adult birds and human hepatoma cells, that type II sites accumulate slowly until they reach 10 to 20% of their fully induced levels, after which their rate of accumulation increases sharply (38). In the adult, this level is reached within 8 to 10 h. In embryos treated at day 13, qualitatively similar behavior was observed, but these critical levels were not reached until 2 to 3 days after stimulation. These experiments also revealed that contrary to all previous data from studies with chicks and adult birds, the expression of both apoVLDLII and vitellogenin genes could be dissociated from the maintenance of elevated levels of nuclear type I receptor.

It is clear from experiments with adult birds that the maintenance of type II sites after withdrawal or metabolism of 17β-estradiol did not, under normal circumstances, result in continued expression of the apoVLDLII and vitellogenin genes. The fact that transcription of both genes was sustained until hatching in embryos treated with estrogen at day 13 raises the possibility that other factors in embryonic liver influence their expression. However, persistent expression might also be explained by the presence of a ligand in the egg that is capable of interacting with the type II site and of promoting expression of the genes. Possible candidates for such a ligand, suggested by competition binding studies to be presented elsewhere, might be one of a number of metabolites of 17β-estradiol that have relatively high affinities for the type II sites. The embryo is likely to be exposed to these metabolites for considerably longer than chicks or roosters are, since the metabolites cannot be excreted until hatching. The rapid decline in apoVLDLII and vitellogenin mRNAs after hatching is consistent with such a proposal. A corollary of this hypothesis is that it should be possible to maintain the expression of the apoVLDLII and vitellogenin genes with type II-specific ligands. Experiments are currently in progress to test this possibility.

Model for the regulation of type II binding sites. In formulating a working model for future studies on the regulatory properties of type II sites, we have taken into consideration the following observations, reported here and derived from previous investigations with human hepatoma cells (37, 38). In HepG2 cells, type II sites can be induced by concentrations of estrogen adequate to saturate the classical high-affinity receptor, but their induction lags behind that of the type I receptor by several hours and is not altered by increasing the concentration of estrogen to a range that would be expected to saturate the type II sites themselves. These observations are consistent with the suggestion that the induction of the type II sites is mediated initially via the type I receptor and is independent of the occupancy of the type II sites by estradiol. The slow initial accumulation of type II sites is followed by a 5- to 10-fold increase in the accumulation rate during the period when their levels are between 10 to 20% of the fully induced levels. In vitro, the withdrawal of hormone during the lag period results in little or no induction of type II sites over the next 24 h, while withdrawal at later times has little effect on their kinetics of accumulation. Both in vivo and in vitro, type II sites, once induced, persist and can be propagated in the absence of estrogen through repeated rounds of cell division. This type of behavior may be explained most simply by postulating that the type II binding sites positively regulate their own synthesis.

The model we propose to explain the regulation of expression of the type II receptor is fundamentally identical with one of several regulatory circuits suggested to explain developmental switching (44), and it is the simplest circuit that will accommodate the experimental observations. We have used computer simulation to test the model and to compare theoretical predictions of this model with our experimental observations (Fig. 4).

We suggest that the increase in the concentration of type I receptor-estrogen complex (T1) induced by the primary administration of estrogen results in increased binding of the receptor to a specific DNA-binding site, or response element, designated RE1. Occupancy of this element stimulates the expression of the gene encoding the type II receptor with an efficiency a_1. As the concentration of type II receptors (T2) increases, as a consequence of expression of the type II gene, they in turn become available for interaction with a second response element designated RE2. Binding of the type II receptor to this element is independent of estrogen and stimulates expression with an efficiency a_2; the overall activity of the gene being the sum of the activation attributable to each element. Interaction of type II receptors with RE2 constitutes a positive-feedback, closed loop that accounts for the accelerated accumulation of type II receptors seen following the initial lag phase, as well as the decreasing dependence of their rate of accumulation on the concentration of occupied type I receptors beyond a certain stage of induction. To demonstrate some of the behavioral characteristics predicted by the model, we present simulated ex-

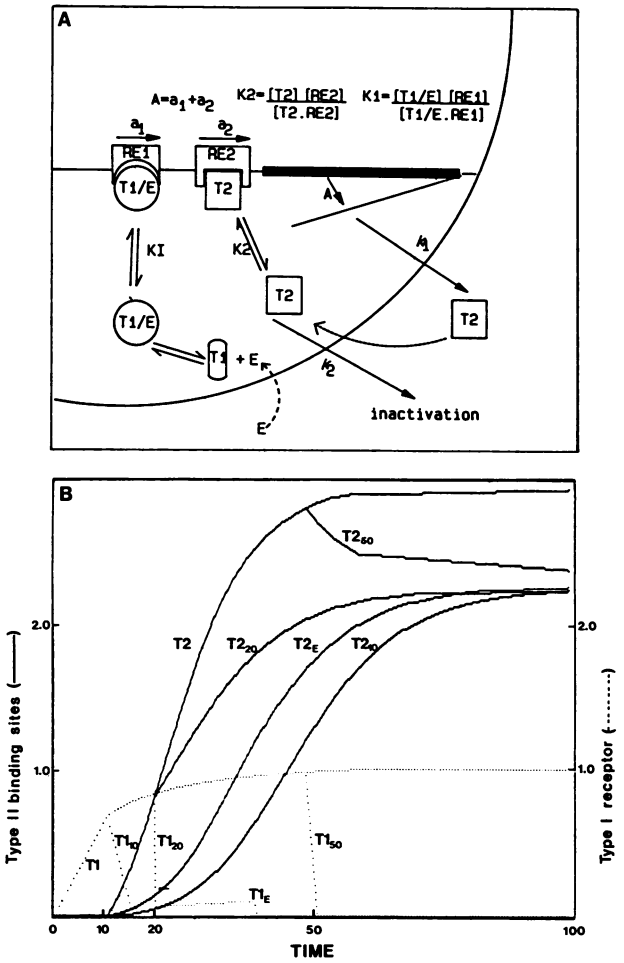

FIG. 4. A model to explain the induction characteristics of type II sites. (A) The assumption has been made that stimulation from each of the two response elements RE1 and RE2 is directly proportional to the extent of occupancy of these elements by their respective ligands, i.e., type I receptor (T1) or type II binding site (T2). In addition, it was assumed that when both elements are occupied, the total activity that results is the summation of the activation attributable to each of the individual elements. Thus, total activity of the gene (A) = stimulation from RE1 + stimulation from RE2, and the stimulation from RE1 = $a_1 \cdot T1/K1 + T1$, where a_1 is a coefficient of activation attributable to RE1, and $K1$ is the dissociation constant for occupied type I receptor binding to RE1, defined as $K1 = [(T1)(RE1)]/(T1 - RE1)$; similarly, stimulation from RE2 = $a_2 \cdot T2/(K2 + T2)$. The overall rate of accumulation of type II sites = $k_1A - k_2T2$, where k_1 and k_2 are intrinsic constants for the production and decay of type II sites respectively. Thus, $dT2/dt = k_1a_1[T1/(K1 + T1)] + k_1a_2[T2/(K2 + T2)] - k_2T2$. A detailed description of the approach used to solve this equation is presented in Materials and Methods. (B) To generate the curves presented, the following relative values were assigned to the constants: k_1, 1.0; k_2, 0.2; $K1$, 3.0; $K2$, 3.0; a_1, 0.25; a_2, 1.0 These values were selected to produce a lag period, kinetics of approach to steady state, and ratio of type I receptors to type II binding sites that approximated relative experimental values. The concentration of type I receptor (-----) was varied with time and was approximated by using the following hyperbolic function for the adult bird: T1 = T1(initial) + 1 $[t/(7 + t)]$, where T1(initial) = 0.01, and T1 = T1(initial) + 0.2 $[t/(49 + t)]$ for day 13 embryos. To evaluate the effect of eliminating the contribution of the type I receptor (T1) on the subsequent accumulation of type II receptor (T2), the T1 value was set to 0 at 10 (curves $T1_{10}$ and $T2_{10}$), 20 (curves $T1_{20}$ and $T2_{20}$), and 50 (curves $T1_{50}$ and $T2_{50}$) arbitrary time units or not set to zero at all (curves T1 and T2). Curves $T1_\varepsilon$ and $T2_\varepsilon$ represent induction in day 13 embryos.

amples of the consequences of withdrawing estrogen (i.e., effectively removing the stimulus from type I receptor) at various times after induction and of altering the rate and extent of accumulation of type I receptor (Fig. 4).

The plots shown illustrate the following points. (i) The model simulates the slow initial phase of type II receptor accumulation seen both in vivo and in cultured cells (curve T2). (ii) Decreasing the rate and extent of the accumulation of type I receptor to mimic the situation in day 13 embryos (curve $T1_\varepsilon$) predicts the marked increase in lag period observed experimentally (curve $T2_\varepsilon$). (iii) Removal of the stimulation from type I receptor prior to a significant increase in the level of type II receptors (curve $T1_{10}$) also markedly increases the lag period (curve $T2_{10}$). (iv) Removal of the stimulus from type I receptors after type II receptors have increased to 15 to 20% (curve $T1_{20}$) has only a slight effect on the accumulation rate (curve $T2_{20}$). (v) Removal of the type I stimulus at the approach to steady state (curve $T1_{50}$) results in a relatively rapid decline to a new steady state maintained by stimulation attributable to type II sites alone (curve $T2_{50}$), a situation that mimics to some extent the induction of type II sites in adult birds by administering a single bolus of hormone.

Intrinsic to this simple model is the prediction that once a finite accumulation of type II receptors has occurred, no matter how small, accumulation of these sites will proceed in the absence of continued stimulation from type I receptors, albeit with extremely long lag times. The experimental data are not yet sufficiently refined to determine whether this is the case or whether the autogenous component of the induction of type II receptors displays a true threshold phenomenon. However, as will be described in detail elsewhere, modification of the model to include multiple response elements for type II receptors, non-first-order decay kinetics, or a requirement for aggregation of subunits to produce an active type II receptor (21) accommodates the existence of a threshold.

The role that type II receptors play in the primary estrogen response may be explained if, when occupied by an appropriate ligand, they augment the rate of expression of a set of target genes whose initial activation is, under normal circumstances, regulated primarily by the type I receptor-estrogen complex. Since the accumulation of type II sites is relatively delayed during primary induction, the influence of these sites on expression would be most apparent for genes that are least responsive to the increase in type I receptor. In the case of avian liver, the vitellogenin gene as opposed to the apoVLDLII gene might be expected to fall into this category. The persistence of type II receptors after the withdrawal of hormone would allow them to influence, to a greater or lesser extent, the early response characteristics of both genes after secondary stimulation. The data obtained from studies with embryos raise the additional possibility that occupancy of type II receptors by an as yet unidentified ligand may be sufficient to sustain the continued expression of both the apoVLDLII and vitellogenin genes independently of the levels of nuclear type I receptor.

ACKNOWLEDGMENTS

We thank John Burch for the generous gift of plasmids pSVT13 and pSAP34. We express our appreciation for constructive comments and reading of the manuscript by our colleagues. We are indebted to Marion Webb for assistance throughout the preparation of the manuscript.

This work was supported by grants to R.G.D. from the Medical Research Council of Canada and the Heart and Stroke Foundation

of Ontario and by grants from the Medical Research Council of Canada and the J. P. Bickell Foundation to M.N., S.-P.T. and R.J.G.H. were recipients of a postdoctoral fellowship from the Heart and Stroke Foundation of Ontario and an Ontario Graduate Scholarship, respectively.

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